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Calreticulin on the mouse egg surface mediates transmembrane signaling linked to cell cycle resumption

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Abstract

Calreticulin, a protein best known as an endoplasmic reticulum chaperone, also is found on the extracellular plasma membrane surface of many cell types where it serves as a mediator of adhesion and as a regulator of the immune response. In this report, we demonstrate that calreticulin is present on the extracellular surface of the mouse egg plasma membrane and is increased in the perivitelline space after egg activation. The extracellular calreticulin appears to be secreted by vesicles in the egg cortex that are distinct from cortical granules. An anticalreticulin antibody binds to extracellular calreticulin on live eggs and inhibits sperm–egg binding but not fusion. In addition, engagement of cell surface calreticulin by incubation of mouse eggs in the presence of anticalreticulin antibodies results in alterations in the localization of cortical actin and the resumption of meiosis as indicated by alterations in chromatin configuration, decreases in cdc2/cyclin B1 and MAP kinase activities, and pronuclear formation. These events occur in the absence of any observable alterations in intercellular calcium. These data demonstrate that calreticulin functionally interacts with the egg cytoskeleton and can mediate transmembrane signaling linked to cell cycle resumption. These studies suggest a role for calreticulin as a lectin that may be involved in signal transduction events during or after sperm–egg interactions at fertilization.

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Keywords: Calreticulin; Egg activation; Oocyte; Signal transduction; Cell cycle; Meiosis

Introduction

Calreticulin is best known as a soluble calcium-binding endoplasmic reticulum protein that serves as a molecular chaperone in the folding of glycoproteins. However, calreticulin also has functional roles in many other aspects of cell physiology (Coppolino and Dedhar, 1998; Michalak et al., 1999). Recently, much interest has focused on the role(s) of calreticulin as a cell surface mediator of adhesion and as a regulator of the immune response. Cell surface calreticulin serves as a binding protein for complement C1q, the first component of the classical complement pathway (Sim et al., 1998). Of particular relevance to this report, calreticulin has roles in calcium signaling (Coppolino et al., 1997; Gudz et al., 2002; John et al., 1998; Kwon et al., 2000; Liu et al., 1994), phagocytosis (Asgari and Schmidt, 2003; Gardai et al., 2003; Muller-Taubenberger et al., 2001; Ogden et al., 2001), and integrin-mediated cell adhesion and platelet activation (Elton et al., 2002; Feng et al., 2002).

Calreticulin is found in several different intracellular compartments (endoplasmic reticulum, nucleus, and cytoplasm) and in extracellular compartments (outer surface of the plasma membrane and serum). It is unclear how the protein is differentially localized to these regions. The mature protein (apparent M_r of approximately 60 kDa) contains an N-terminal signal sequence and a C-terminal KDEL endoplasmic reticulum retention sequence, as well as a putative nuclear localization sequence (Coppolino and Dedhar, 1998). Although much calreticulin is retained

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within the endoplasmic reticulum, it is now clear that a subpopulation of cellular calreticulin escapes KDEL-mediated ER retention and becomes localized to the cell surface. There is evidence from several cell types that this localization is mediated by the secretory pathway (Johnson et al., 2001).

Calreticulin associates with the extracellular plasma membrane surface via interactions with adaptor proteins (Arosa et al., 1999; Ghiran et al., 2003). Calreticulin also interacts with the extracellular domains of integrins (Elton et al., 2002; Feng et al., 2002; Zhu et al., 1997), but it is unknown if this is a direct or indirect interaction. It has been postulated that interactions with different adaptor proteins could explain the diverse physiological effects of extracellular engagement of surface calreticulin (Ghiran et al., 2003).

Sperm–egg interactions at fertilization have striking parallels to cell–cell adhesion-mediated cell activation events that occur during immune responses. Ligation of immune cell adhesion receptors leads to phospholipase C activation, calcium signaling, alterations in transcriptional regulation, cytoskeletal reorganization, phagocytosis, and exocytosis (Sims and Dustin, 2002). Fertilization results in similar cell activation events, e.g., calcium oscillations, engulfment of the sperm by the egg, and exocytosis of cortical granules. In the case of immune cells, several different types of adhesion receptors can initiate these pathways; these include integrins, members of the immunoglobulin superfamily, and C-type lectins (Sims and Dustin, 2002). At least one A disintegrin and metalloprotease (ADAM) protein is expressed predominantly on lymphocytes and may have a role in lymphocyte–leukocyte adhesion (Bridges et al., 2002), and tetraspan proteins can modulate cell interactions important during the immune response (Fearon and Carter, 1995; Flint and McKeating, 2000). It is unclear exactly what molecule(s) on the egg surface mediates adhesive interactions with sperm. To date, the best evidence is that an egg plasma membrane tetraspanin protein, CD9, is critical for sperm–egg fusion (Le Naour et al., 2000; Miller et al., 2000; Miyado et al., 2000). A number of laboratories have published data supporting a role for integrin β1 in sperm–egg binding (Evans, 2002; Nishimura et al., 2001; Zhu and Evans, 2002), but a role for any integrins at all in sperm–egg interactions recently has been questioned (He et al., 2003). There is good evidence for a role for sperm surface ADAM family proteins in sperm–egg binding (Evans, 2002; Nishimura et al., 2001; Zhu and Evans, 2002). It has been suggested that a "tetraspan web" on the egg plasma membrane surface that contains tetraspanin proteins and integrins may be important for sperm–egg interactions (Takahashi et al., 2001). The obvious parallels between sperm–egg interactions and immune cell activation raise the possibility that molecules such as calreticulin that are important in certain immune responses also could mediate specific aspects of egg activation at fertilization.

Calreticulin is found on the extracellular surface of hamster and mouse eggs (Calvert et al., 2003; Munoz-Gotera et al., 2001). Based on the above roles for calreticulin, and the similarities between fertilization and immune cell activation, we hypothesized that mouse egg surface calreticulin is involved in egg activation at fertilization. In fact, calreticulin-null *C. elegans* mutants are subfertile, having defects in both male germ cell and oocyte development (Park et al., 2001). Calreticulin-null mice have been generated by homologous recombination, but because there is an embryonic lethal failure of cardiac development, a role for calreticulin in reproduction could not be evaluated (Mesaeli et al., 1999).

In this report, we demonstrate that calreticulin is present on the extracellular surface of the egg plasma membrane and is increased in the perivitelline space of mouse eggs after fertilization. Affinity-purified antibodies bind to extracellular calreticulin on live eggs and decrease sperm binding to eggs lacking a *zona pellucida* (ZP), but do not inhibit in vitro fertilization of eggs with or without a ZP. However, incubation of eggs in the presence of two different affinity-purified antibodies raised against distinct regions of calreticulin results in alterations in the localization of cortical actin and the resumption of meiosis, suggesting that calreticulin functionally interacts with the egg cytoskeleton and is capable of mediating cell cycle resumption. These studies suggest a role for calreticulin as a lectin that may be involved in signal transduction events during or after sperm–egg interactions at fertilization.

Materials and methods

Materials

Female CF-1 mice were from Harlan Sprague–Dawley (Wilmington, MA); males were B6SJLF_1/J or B6D2F_1/J from Jackson Laboratory (Bar Harbor, ME). Primary antibodies used were affinity purified goat antimouse calreticulin (T-19; Santa Cruz Biotechnology, Santa Cruz, CA), raised against a peptide from the amino terminus of mouse calreticulin, affinity-purified goat antimouse calreticulin (C-17; Santa Cruz Biotechnology), raised against a peptide from the carboxyl-terminus of mouse calreticulin, nonimmune goat IgG (Jackson Immunoresearch, West Grove, PA), rabbit antigoat Alexa Fluor™ 488 and rabbit antimouse Alexa Fluor™ 488 (Molecular Probes, Eugene, OR). All other reagents were from Sigma (St. Louis, MO) unless otherwise indicated.

Oocyte, egg, and embryo collection and culture

Standard collection medium was Whittens medium (Whitten, 1971) supplemented with 0.01% polyvinyl alcohol (PVA) and 15 mM HEPES, pH 7.2 (W/H/PVA). Culture media used were Whittens medium sup-

plemented with 0.01% PVA (W/PVA) or CZB (Specialty Media, Phillipsburg, NJ). Fully grown, germinal vesicle (GV)-intact oocytes were collected as previously described (Schultz et al., 1983). To prevent GV breakdown, 0.25 mM dibutyryl cyclic AMP (dbcAMP) was included in the oocyte collection and culture medium (Cho et al., 1974). Metaphase II-arrested eggs were collected from superovulated mice 14–15 h after hCG administration and the cumulus cells were dispersed by treatment with 0.1% hyaluronidase as previously described (Manejwala et al., 1989). One-cell embryos were flushed from the oviducts of superovulated, mated females 18–20 h after hCG administration. When necessary, the zona pellucida (ZP) was removed by brief incubation in acid Tyrode's medium (pH 1.6). For all experiments, cells were cultured under light mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air.

Immunofluorescence procedures

ZP-free cells were fixed in 3.7% paraformaldehyde, permeabilized in 0.1% TX-100, and processed for immunofluorescence as previously described (Evans et al., 1995). When the ZP was present, the same procedure was performed except that the cells were washed overnight after incubation in the secondary antibody to reduce nonspecific ZP staining. Antibodies used were anticalreticulin T-19 or C-17, or nonimmune goat IgG used at a final concentration of 4 μg/ml, and the secondary antibody was antigoat Alexa Fluor™ 488 (4 μg/ml; Molecular Probes). When live cells were stained, the antibodies first underwent buffer exchange to remove sodium azide (see below). The primary antibodies then were used at a final concentration of 150 μg/ml in W/H/PVA. Live cells were immunostained for 2 h at 37°C or 4°C. Cortical granules were stained using biotin-conjugated lens culinaris agglutinin (10 μg/ml; Vector Laboratories, Burlingame, CA) followed by avidin-Texas Red (5 μg/ml, Molecular Probes). Tubulin was stained using a monoclonal anti-β tubulin antibody (2 μg/ml) followed by antimouse Alexa Fluor™ 488 (4 μg/ml, Molecular Probes). To stain DNA, cells were mounted in Vectashield (Vector Laboratories) containing 1.5 μg/ml DAPI. Cells were observed using a Leica TCS 4D laser-scanning confocal microscope.

Electrophoresis and immunoblotting

ZP-intact oocytes were placed directly into sample buffer, denatured, separated on reducing SDS-polyacrylamide gels (Laemmli, 1970), and then electrophoretically transferred to Immobilon™-P (Millipore, Bedford, MA). Primary antibodies (T-19, C-17, and nonimmune goat IgG) were used at a final concentration of 0.4 μg/ml. The secondary antibody was alkaline phosphatase-conjugated antigoat IgG used at 0.4 μg/ml (Jackson ImmunoResearch). Immunoreactive proteins were detected using VISTRA™ reagent (Amersham, Arlington Heights, IL), and visualized using a Molecular Dynamics STORM™ system (Amersham Biosciences, Sunnyvale, CA).

Live cell treatments

Antibodies used for experiments involving live cells underwent a buffer exchange procedure to remove sodium azide as follows. Approximately 300 μg of each antibody was concentrated into a volume of 30 μl in a Centricon-30 concentrator (Millipore). The antibody was resuspended in 500 μl PBS, concentrated again, and resuspended as before in 500 μl PBS. The antibodies were concentrated a final time to 10–30 mg/ml and stored at −20°C until use. The final protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL). Eggs were placed in microdrops containing T-19, C-17, or nonimmune IgG at 0.5–5 mg/ml in CZB and incubated at 37°C for up to 48 h, as indicated. For some experiments, eggs were incubated in the presence of 0.2–200 μM PD 98059, 10–50 μM LY 294002 (Upstate Biotechnology, Lake

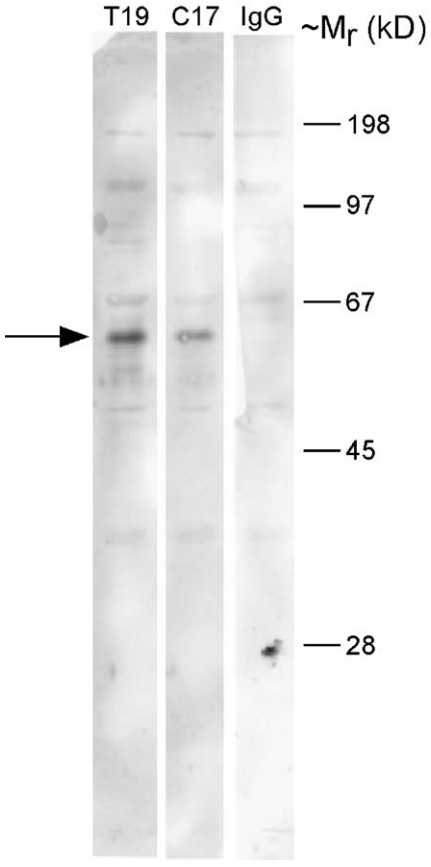

Fig. 1. Calreticulin antibody recognizes a single immunoreactive band in mouse eggs. Immunoblot of 100 eggs per lane (approximately 2.5 μg protein) probed with anticalreticulin T-19, anticalreticulin C-17, or nonimmune IgG, as indicated. Arrow indicates the immunoreactive band of approximately 60 kDa in the anticalreticulin lanes. This experiment was done twice; a representative immunoblot is shown.

Placid, NY), or 1–2 μM bisindolylmaleimide I (BIM; Calbiochem, San Diego, CA) during the antibody incubation period. Original stock solutions of these three reagents were prepared with DMSO and the final working dilutions were made in CZB; an equivalent amount of DMSO was added to control media.

In vitro fertilization

In vitro fertilization of ZP-intact eggs was performed as described (Moore et al., 1993). Briefly, sperm obtained from $B6SJLF_1/J$ males were capacitated for 2 h in Whittens medium containing 15 mg/ml BSA (Albumax I; Life Technologies, Rockville, MD). Metaphase II-arrested eggs were incubated in the presence of T-19 or nonimmune IgG (5 mg/ml in CZB) for 60 min. The eggs then were washed to remove excess antibody and placed into 250 μl fertilization drops of Whittens medium containing 15 mg/ml BSA and 2×10^5 sperm/ml. The eggs were coincubated with sperm for 3 h, then washed free of unbound sperm. The eggs were fixed, permeabilized, and then stained for DNA by mounting in Vectashield (Vector Laboratories) containing 1.5 μg/ml DAPI. Fertilization was evaluated by examining the eggs for the presence of decondensing sperm heads using an epifluorescence-equipped Nikon TE-300 microscope.

For in vitro fertilization of ZP-free eggs, the ZPs were removed as described above and the eggs were allowed to recover by incubation in CZB for 60 min before use. After the recovery time, the eggs were incubated in the presence of T-19 or nonimmune IgG (5 mg/ml in CZB) for 60 min. The eggs were washed to remove unbound antibody, then placed into fertilization drops containing 10^5 sperm/ml for 60 min. Loosely bound sperm were washed off of the eggs; the same pipette (approximately 90-μm diameter) and number of washes was performed for each group. The eggs were stained with DAPI as above, and the number of sperm bound or fused per egg was determined.

Calcium imaging

Eggs were incubated for 20 min in fura-2 acetoxymethyl ester (Fura-2; 10 μM in W/PVA containing 0.025% Pluronic F-127; Molecular Probes). The eggs were washed, then placed on a temperature-controlled microscope stage in 2-μl drops of CZB under mineral oil and laminar flow of 5% CO_2 in air. After baseline measurement of intracellular calcium level, 2 μl of either anticalreticulin T-19 or nonimmune goat IgG was added to the eggs for a final antibody concentration of 2–5 mg/ml, and intracellular calcium levels were monitored for 1–6 h. As a control for the

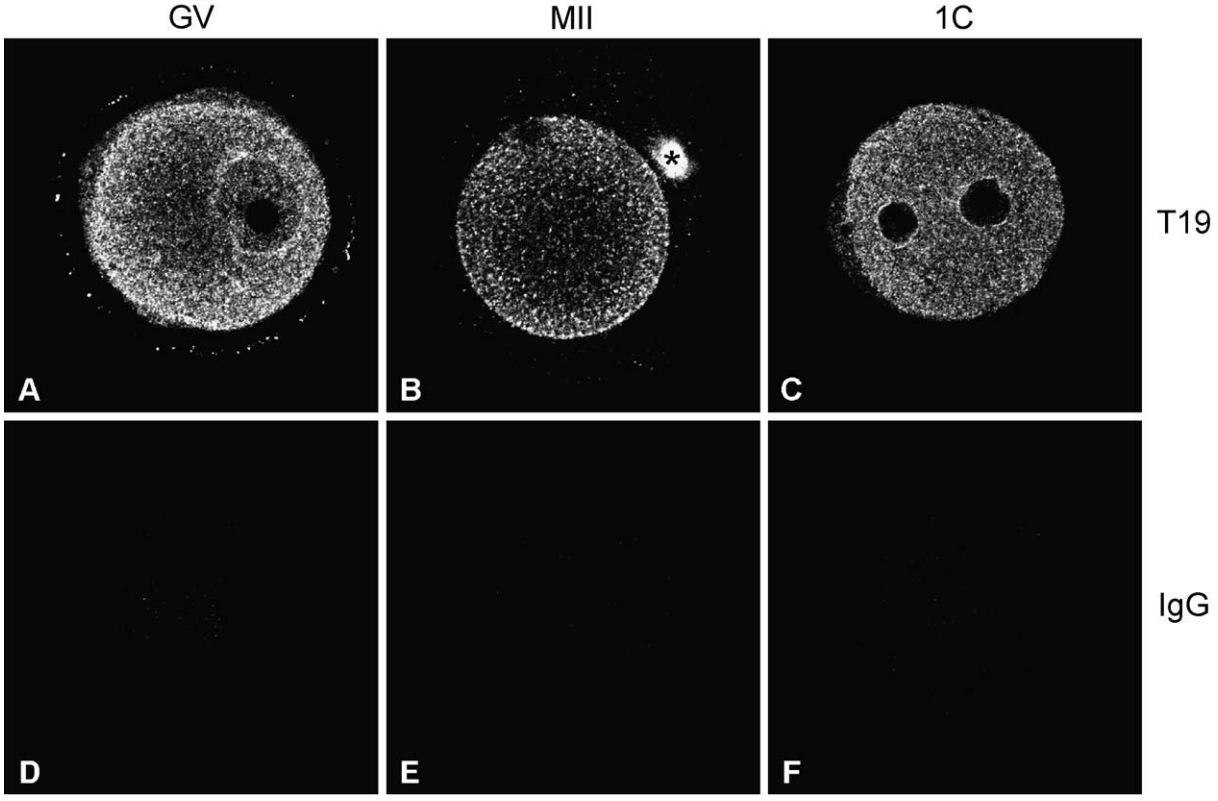

Fig. 2. Localization of calreticulin in ZP-intact oocytes, eggs, and one-cell embryos by confocal microscopy. Fixed, permeabilized cells were immunostained with anticalreticulin T-19 (A–C) or nonimmune IgG as a control (D–F). (A, D) GV, GV-intact oocytes. (B, E) MII, metaphase II-arrested eggs. (C, F) 1C, one-cell embryos. Asterisk indicates nonspecific staining of the polar body that is seen with many primary and secondary antibodies. At least 12 cells were examined in each group in three independent experiments, and representative examples are shown.

calcium imaging procedure, eggs were washed after 6 h and then monitored for intracellular calcium during treatment with 10 mM strontium chloride in calcium- and magnesium-free CZB. For some experiments, eggs were loaded with Fura-2 as above after incubation for 6 h in 2 mg/ml T-19, 2 mg/ml nonimmune IgG, or CZB alone, and then placed back into the original microdrops of antibody or medium. Intracellular calcium levels were monitored for the next 10 h in the continuous presence of the same antibody or medium alone. Measurement of intracellular calcium oscillations was performed using a filter wheel to alternate excitation wavelengths between 340 and 380 nm and measuring the emission ratios through a Fura-2 band pass filter cube; ratios were recorded at 10-s intervals. Calcium levels were recorded using a Princeton Instruments MicroMAX CCD camera (Roper Scientific, Trenton, NJ) and analyzed using MetaFluor software (Universal Imaging Corp., Downingtown, PA).

Kinase assays

The cdc2/cyclin B1 and MAP kinase activities in individual eggs were measured by assays of the phosphorylation of histone H1 and myelin basic protein (MBP), respectively, as previously described (Svoboda et al., 2000). The peak value of histone H1 or MBP kinase activity in the IgG group was arbitrarily set at 100%, and the values obtained in the other groups of eggs were expressed relative to this amount. Using the peak rather than the mean value allowed for the possibility that some of the IgG-treated eggs could have begun cell cycle resumption; this peak value was not different from that observed previously in metaphase II eggs.

Results

Calreticulin localization in mouse oocytes, eggs, and one-cell embryos

To confirm the specificity of the antibodies used in the subsequent experiments, immunoblot analysis of oocytes was performed using two different polyclonal anticalreticulin antibodies. The T-19 antibody was raised against a portion of the N-terminal region of calreticulin, and the C-17 antibody was raised against a portion of the C-terminus. Both antibodies recognized a single immunoreactive band of approximately 60 kDa, consistent with the expected size of calreticulin, which was not recognized by nonimmune IgG (Fig. 1).

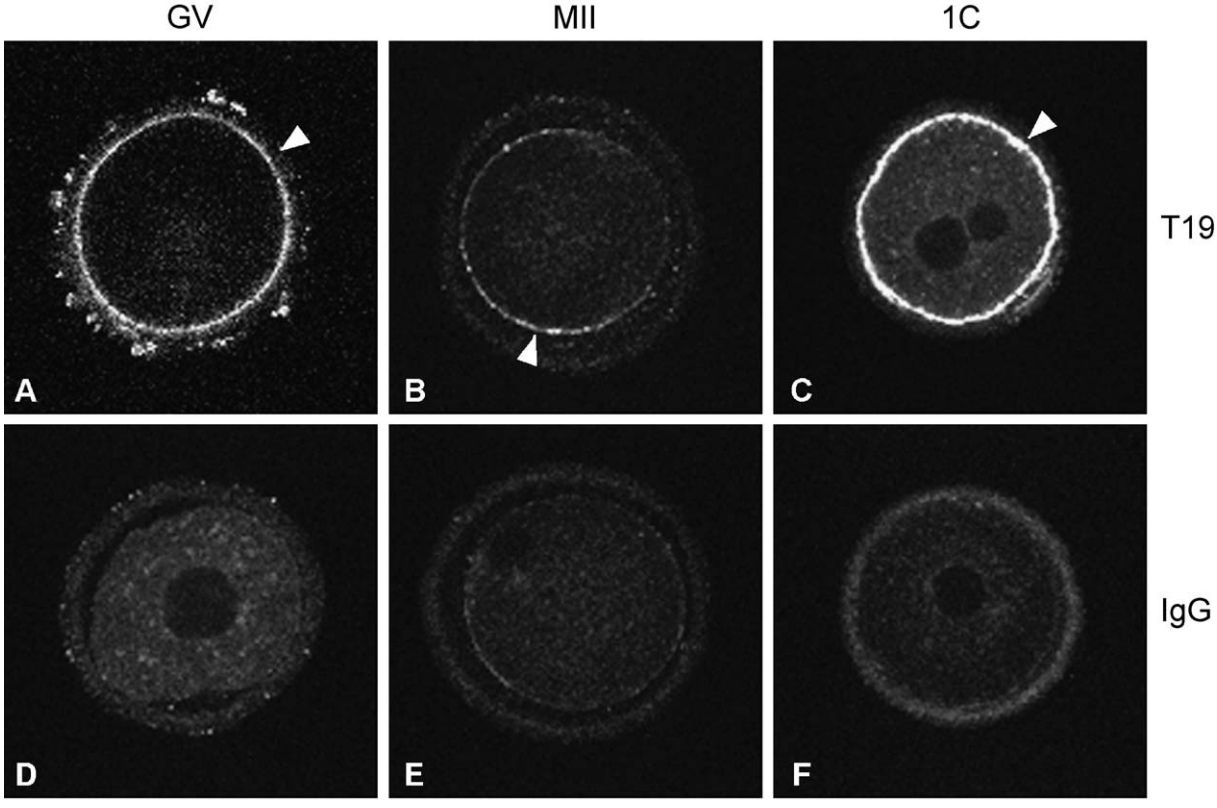

Fig. 3. Localization of calreticulin on the extracellular surface of ZP-intact oocytes, eggs, and one-cell embryos by confocal microscopy. Live cells were immunostained with anticalreticulin T-19 (A–C) or nonimmune IgG (D–F). (A, D) GV, GV-intact oocytes. (B, E) MII, metaphase II-arrested eggs. (C, F) 1C, one-cell embryos. Arrowheads in panels A–C indicate areas of enhanced fluorescence beneath the ZP and at the plasma membrane. At least 15 cells were examined in each group in three independent experiments, and representative examples are shown.

Localization of calreticulin was determined in permeabilized, ZP-intact oocytes, eggs, and one-cell embryos by confocal microscopy. Calreticulin was found in a somewhat punctate pattern throughout the cytoplasm, consistent with staining of the endoplasmic reticulum (Figs. 2A–C). In the oocyte, calreticulin staining was enhanced in the cortical/plasma membrane region and in the region immediately surrounding the GV. A lower intensity of staining was seen within the GV and in the perivitelline space. In the egg, calreticulin staining was particularly enhanced in the cortical/plasma membrane region. In the one-cell embryo, an enhancement of staining was seen surrounding both the male and female pronuclei that was similar to that around the oocyte GV, but there was no staining within the pronuclei. Similar to the oocyte, there was a lower intensity of staining in the perivitelline space. Cells stained using control nonimmune IgG exhibited no staining (Figs. 2D–F).

The membrane region and perivitelline space staining seen in the permeabilized cells was consistent with previous work, suggesting that calreticulin was present on the egg surface, and possibly secreted after egg activation (Calvert et al., 2003; Munoz-Gotera et al., 2001). To definitively determine if calreticulin was present on the extracellular surface of the plasma membrane, living oocytes, eggs, and one-cell embryos were incubated in the presence of the T-19 antibody or control nonimmune IgG. T-19 stained the perivitelline space below the ZP, with the staining significantly brighter in the one-cell embryo as compared to both oocytes and eggs (Figs. 3A–C); the C-17 antibody had a similar staining pattern (data not shown). This staining pattern was not seen when the primary antibody was nonimmune IgG (Figs. 3D–F). In addition, cell surface staining for calreticulin was minimal when the ZP was removed before incubation with the primary antibody (data not shown).

Calreticulin does not colocalize with cortical granules

At fertilization, cortical granules (CG) undergo exocytosis and release their contents into the perivitelline space. We hypothesized that calreticulin might be found within the mouse egg CGs because the calreticulin staining in the perivitelline space significantly increased in the one-cell embryo as compared to the egg. Eggs were costained for both calreticulin and CGs, and then analyzed by confocal microscopy. In confocal sections through the equatorial plane, the CG staining was generally external to calreticulin in the cortex (Fig. 4A). Confocal sections through the cortex

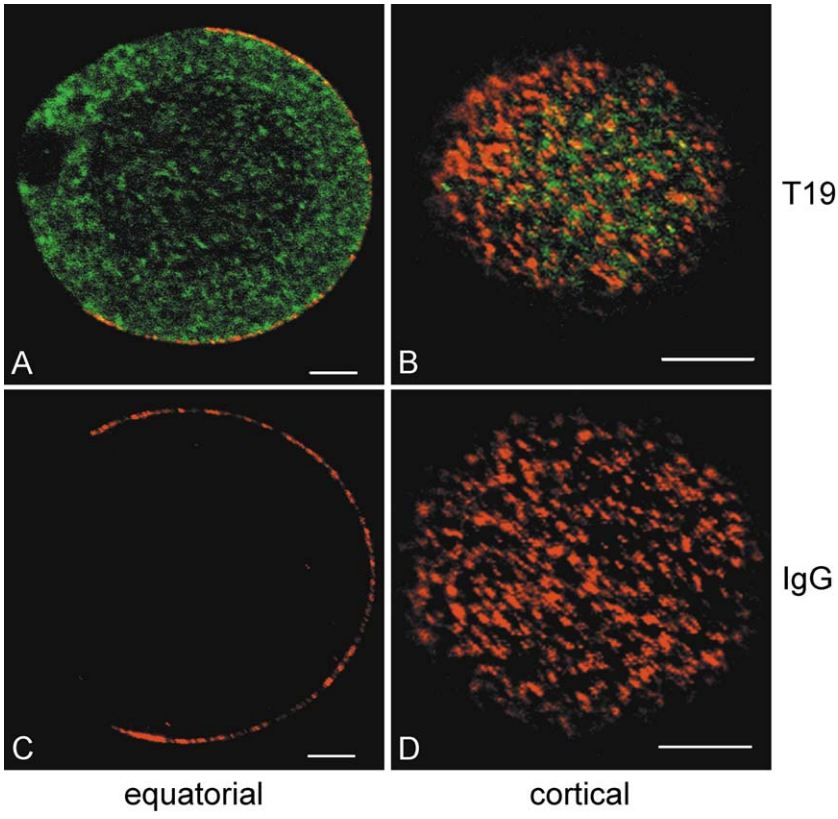

Fig. 4. Calreticulin does not colocalize with cortical granules. Eggs were stained for cortical granules using lens culinaris agglutinin (A–D; red). In addition, the eggs were immunostained with either primary anticalreticulin T-19 antibody (A, B) or nonimmune goat IgG (C, D) followed by antigoat Alexa Fluor™ 488 (green). Panels A and C are sections through the equatorial region, and panels B and D are sections through the cortical region. The calculated optical thickness of the confocal sections was 0.7 μm. Scale bar, 10 μm. A total of 10 eggs in two independent experiments were viewed on a confocal microscope, and representative images are shown.

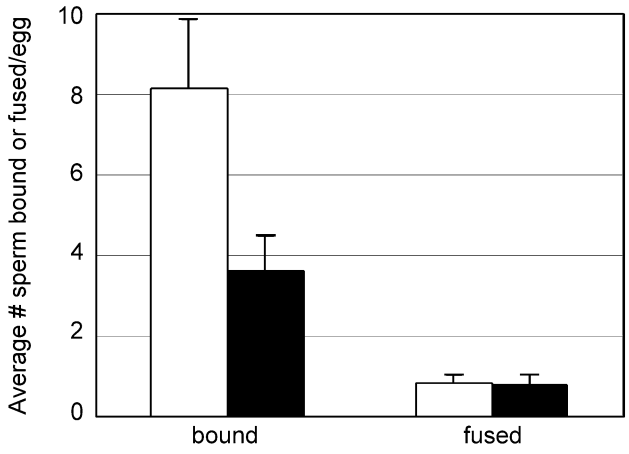

Fig. 5. Effect of anticalreticulin antibody on sperm–egg binding and fusion during ZP-free in vitro fertilization. ZP-free eggs were incubated in the presence of 5 mg/ml anticalreticulin T-19 antibody (black bars) or nonimmune goat IgG (white bars) for 2 h. The eggs then were washed and inseminated. After 60-min coincubation with sperm, the eggs were washed free of unbound sperm, fixed, and stained using DAPI. The total number of sperm bound and fused with each egg was determined and expressed as an average per egg. Shown is the mean ± SE of eight independent experiments. The average number of sperm bound in the T-19 and IgG groups were significantly different (Mann Whitney U test, $P < 0.05$).

revealed that although both calreticulin and CGs were present, their localization was almost completely distinct (Fig. 4B). Eggs stained with control nonimmune IgG instead of the T-19 antibody had essentially no background signal, and only exhibited a CG staining pattern (Figs. 4C–D). These data demonstrate that calreticulin and CGs are located close to each other in the egg cortex but are distinct.

Effect of calreticulin antibodies on in vitro fertilization

The localization of calreticulin on the extracellular surface of the egg suggested that this protein could be directly involved in sperm–egg interactions at fertilization. To test this hypothesis, ZP-free eggs were incubated in T-19 antibody or nonimmune IgG before insemination, and then assayed for sperm binding and fusion. The average number of sperm bound per egg was significantly reduced after incubation in the presence of the T-19 antibody as compared to nonimmune IgG (Fig. 5). However, the number of sperm fused per egg was no different between the two treatment groups. Similarly, incubation of ZP-intact eggs in the T-19 antibody did not affect fertilization (as indicated by sperm–egg fusion) when compared to control eggs incubated in

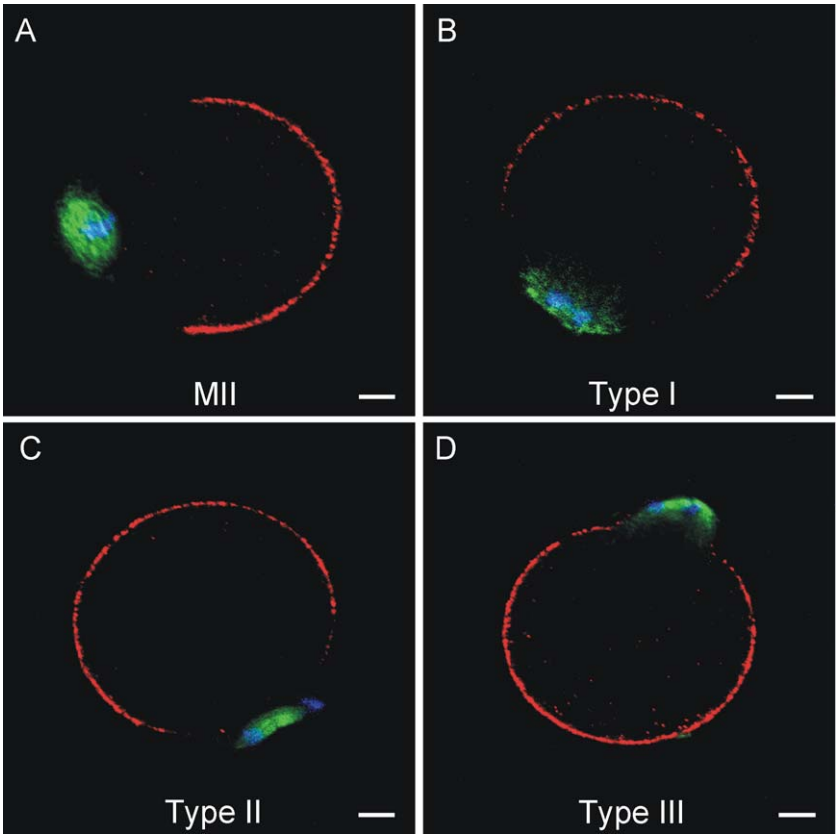

Fig. 6. Effect of anticalreticulin antibody treatment of eggs on chromatin configuration and cortical granule distribution. Eggs were treated with two different anticalreticulin antibodies (T-19, N-terminal; C-17, C-terminal) or goat IgG. After 8 h, the eggs underwent ZP removal and then were stained for cortical granules, tubulin, and DNA. Representative images of four different chromatin and cortical granule configurations are shown in panels A–D. (A) Metaphase II (chromatin at metaphase plate, large CG-free domain). (B) Type I (chromatin in early anaphase, large CG-free domain). (C) Type II (late anaphase, smaller CG-free domain). (D) Type III (chromatin present in polar body-like structure, no CG-free domain).

Table 1
Summary of DNA and cortical granule configuration of eggs treated with anticalreticulin antibodies or nonimmune IgG

Antibody	Concentration (mg/ml)	DNA/cortical granule configuration				Total evaluated	Percentage of eggs not displaying MII configuration
		MII	Type I[a]	Type II[a]	Type III[a]		
T-19	5	0	2	4	16	22	100
	2	8	11	10	9	38	79
	1	4	4	7	9	24	83
	0.5	14	6	3	3	26	46
C-17	5	2	3	8	12	25	92
	2	5	10	10	9	34	85
	1	10	5	2	4	21	52
	0.5	22	2	0	0	24	8
Goat IgG	5	23	0	0	0	23	0
	2	43	0	0	0	43	0
	1	13	0	0	0	13	0
	0.5	14	0	0	0	14	0

[a] Types I, II, and III DNA/cortical granule configurations correspond to those shown in Figs. 6B, C, and D, respectively.

nonimmune IgG or medium alone (data not shown). These results demonstrate that the T-19 antibody reduces sperm–egg binding, but that once binding has occurred it does not interfere with sperm–egg fusion.

Exposure of mouse eggs to calreticulin antibodies induces resumption of the cell cycle

During the course of the in vitro fertilization experiments, it became apparent that treatment of noninseminated eggs with anticalreticulin antibodies resulted in the resumption of meiosis as indicated by the progression of the egg chromatin to an anaphase configuration, and in some cases the extrusion of a polar body-like structure. In fact, cell cycle resumption with the induction of an egg plasma membrane block to polyspermy (McAvey et al., 2002; Wolf, 1978; Yanagimachi, 1994) was one possible explanation for the reduction in sperm–egg binding seen in response to the T-19 antibody. To more carefully define this phenomenon, eggs treated with the T-19 antibody were stained for DNA, tubulin, and cortical granules. In addition, similar experiments were performed using the C-

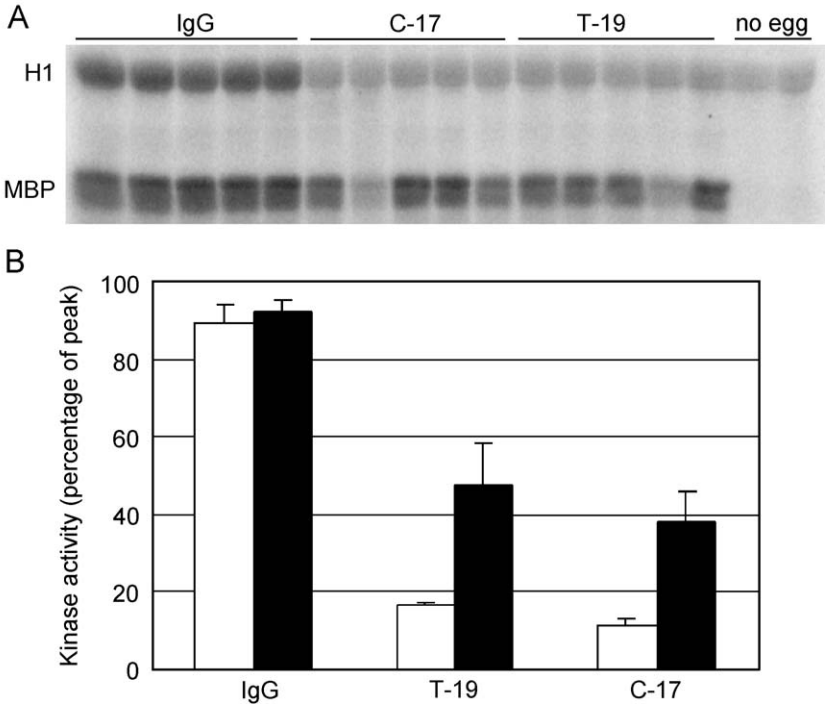

Fig. 7. Effect of calreticulin antibody treatment on cell cycle resumption in eggs. Eggs were treated with two different anticalreticulin antibodies (T-19, N-terminal; C-17, C-terminal) or goat IgG. Individual eggs were washed and then assayed for H1 kinase and MBP kinase activities. (A) Representative kinase assay gel showing the signal obtained from five individual eggs in each treatment group and two control lanes that contained no egg extract. (B) Graph representing the mean ± SE of the H1 kinase (white bars) or MBP kinase (black bars) activity of individual eggs pooled from two different experiments. Activity is expressed as the percentage of the peak activity level for that particular experiment. At least eight eggs were evaluated in each group.

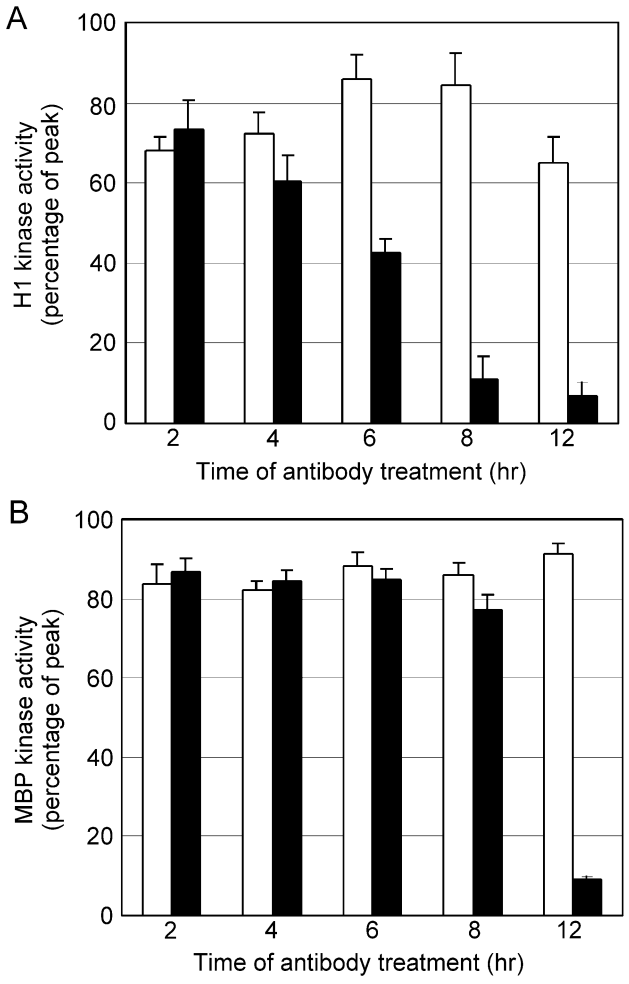

Fig. 8. Time course of the effect of calreticulin antibody treatment on cell cycle resumption. Eggs were treated with goat IgG (white bars) or T-19 antibody (black bars) for the time indicated. Individual eggs were washed and then assayed for H1 kinase (A) and MBP kinase (B) activities. The graphs represent the mean ± SE of the kinase activity of individual eggs pooled from four independent experiments. Activity is expressed as the percentage of the peak activity level for that particular experiment. Six to ten eggs were evaluated in each group.

17 antibody to ensure that the observed phenotype was not a nonspecific effect of the T-19 preparation. Examples of the types of staining seen in these eggs, which were classified as metaphase II (MII) or Type I–III, are shown in Fig. 6. Control metaphase II eggs had the chromatin aligned on the metaphase plate, and cortical granules present only in the microvillar region of the plasma membrane (Fig. 6A). Type I eggs had early anaphase chromatin as seen by a slight separation of the chromatin into two distinct regions; the cortical granule-free domain was still present (Fig. 6B). Type II eggs were in late anaphase, and the CG-free domain was reduced in size with some granules being present in the region of the plasma membrane near the chromatin (Fig. 6C). Type III eggs had extruded a polar body-like structure, and the CG-free domain was restricted to the region directly beneath this structure (Fig. 6D). Based on the morphology of the chromatin and polar body-like structure, there appeared to be a progression of the antibody effect from Type I through Type III configurations.

The concentration dependence of this effect was examined by comparing the chromatin/CG morphology of eggs treated with different concentrations of T-19, C-17, or nonimmune IgG for an 8-h time period. The lowest effective concentration of the anticalreticulin antibodies was 0.5 mg/ml, and the T-19 antibody was more effective than the C-17 antibody (Table 1). In no cases did incubation of eggs for 8 h in nonimmune goat IgG result in alterations in chromatin or CG configuration. Consistent with the idea that the treated eggs were progressing from the Type I through Type III configurations, the lowest concentration of T-19 and C-17 antibodies resulted in very few Type III eggs.

To determine if the antibody treatment was triggering cell cycle resumption, individual eggs were assayed for the levels of cdc2/cyclin B1 and MAP kinase activities after 8 h of treatment. When compared to control eggs, T-19- and C-17-treated eggs had a significant decrease in both kinase activities as measured by phosphorylation of histone H1 and MBP, indicating that cell cycle resumption had occurred (Fig. 7). The time course of cell cycle resumption was measured by assaying eggs treated with the T-19 antibody or nonimmune IgG at time points between 2 and 12 h after initiation of antibody treatment. A significant reduction in H1 kinase activity was seen after 6 h of treatment, while the decrease in MAP kinase activity occurred between 8 and 12 h (Fig. 8). These results demonstrate that cell cycle resumption did not occur until several hours after the initiation of antibody treatment, and that similar to cell cycle resumption at fertilization, the decrease in cdc2/cyclin B1 kinase activity preceded the decrease in MAP kinase activity.

Table 2
DNA configuration of eggs treated for 24 h with anticalreticulin antibodies or nonimmune IgG

Antibody	Antibody concentration (mg/ml)	DNA configuration (% of total eggs)				Total evaluated
		Metaphase	Anaphase–telophase	Pronucleus	Two-cell	
T-19	2	0 (0%)	0 (0%)	11 (42%)	15 (58%)	26
	1	7 (35%)	9 (45%)	0 (0%)	4 (20%)	20
Goat IgG	2	19 (73%)	0 (0%)	4 (15%)	3 (12%)	26
	1	29 (100%)	0 (0%)	0 (0%)	0 (0%)	29
Medium	0	37 (92%)	0 (0%)	1 (3%)	2 (5%)	40

As a more stringent test of the resumption of meiosis, we determined if the eggs that resumed meiosis would complete meiosis II. Eggs were cultured for 24 h in the presence of T-19, nonimmune IgG, or medium alone, then processed for DNA staining using DAPI and analyzed by epifluorescence microscopy. Of the eggs treated with 2 mg/ml T-19, 26/26 (100%) underwent pronuclear formation, and 15/26 (58%) went on to cleave within 24 h (Table 2). Consistent with our previous observations of a concentration-dependent effect of the antibody, a less dramatic effect was seen with 1 mg/ml T-19. In contrast, only 7/26 (27%) of the eggs treated with 2 mg/ml IgG for 24 h formed pronuclei, and 3/26 (12%) went on to cleave, while none of those treated with 1 mg/ml IgG resumed

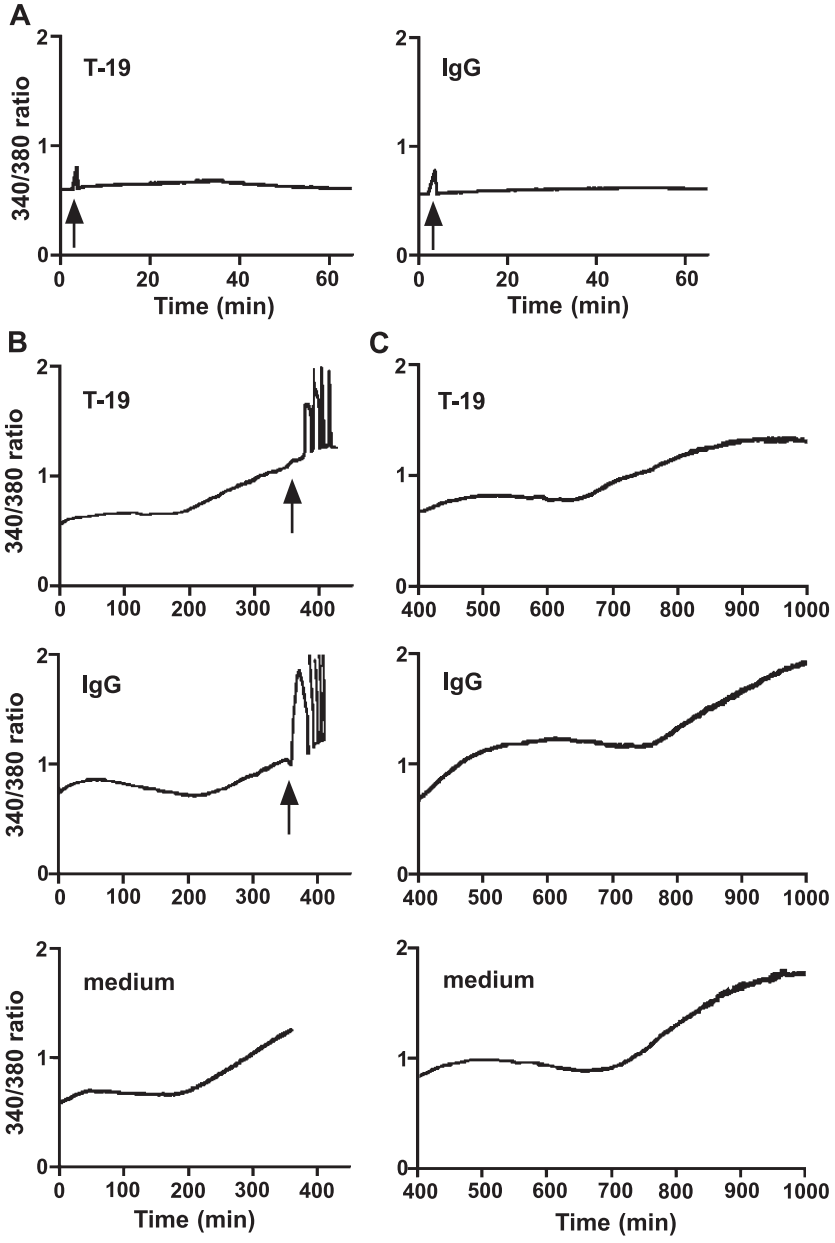

Fig. 9. Effect of calreticulin antibody treatment on intracellular calcium levels. (A) Eggs were loaded with Fura-2 and then baseline calcium measurements were recorded. Either T-19 or nonimmune goat IgG, as indicated, was added to the eggs on the microscope stage to achieve a final concentration of 5 mg/ml. The eggs were monitored for intracellular calcium levels for the next 60 min. Arrows indicate time of antibody addition. (B) Eggs were loaded with Fura-2 and then incubated for 6 h in the presence of T-19 antibody, nonimmune goat IgG, or medium alone, as indicated. During the antibody incubation, intracellular calcium levels were monitored continuously. As a control for the calcium imaging procedure, some of the eggs were washed after 6 h and then monitored for intracellular calcium during treatment with strontium chloride. Arrows indicate the time of strontium activation. (C) Eggs were incubated for 6 h in the presence of T-19 antibody, nonimmune goat IgG, or medium alone, as indicated, and then loaded with Fura-2. Intracellular calcium levels were monitored for the next 10 h in the continuous presence of the same antibody or medium alone. The time indicated on the *X* axes is the time from the beginning of antibody treatment. In all groups, at least 25 individual eggs were monitored in two independent experiments.

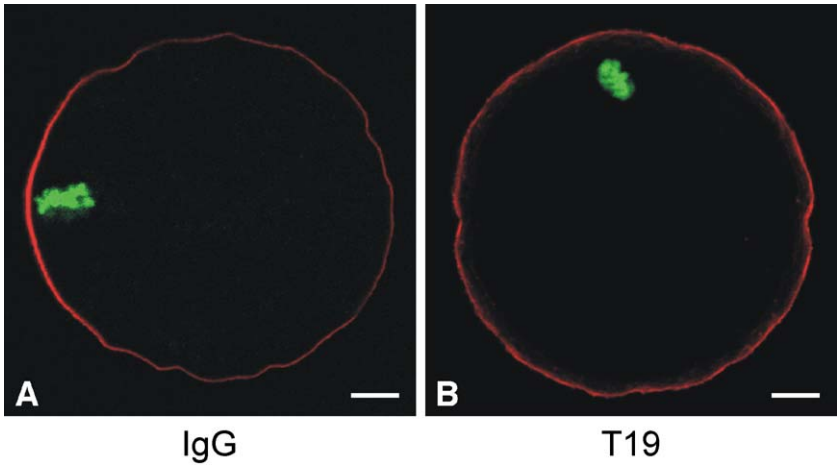

Fig. 10. Effect of calreticulin antibody treatment on actin distribution. Eggs were incubated for 4 h in the presence of goat IgG or T-19 antibody (5 mg/ml) and then fixed and stained for DNA (green) and actin (red). (A) Goat IgG; (B) T-19 antibody. This experiment was repeated twice and representative confocal images are shown.

meiosis. The spontaneous activation rate in eggs kept in medium alone for 24 h was 8%. Of note, culture of these treated eggs for longer periods of time (up to 48 h) only rarely resulted in the formation of any three-cell or four-cell embryos.

Mechanism of induction of cell cycle resumption

The most likely explanation for the resumption of meiosis in the treated eggs was that the antibody treatment was somehow inducing alterations in intracellular calcium levels. To determine if an increase in calcium occurred shortly after treatment of the eggs with antibody, a baseline measurement of intracellular calcium in the eggs was recorded using a ratio imaging system, and then either T-19 or nonimmune IgG was added to the eggs. No increase in intracellular calcium was detected in the treated eggs despite continuous monitoring for 60 min (Fig. 9A). Because the cell cycle resumption response was not observed for several hours following antibody treatment, we also examined the eggs for later alterations in intracellular calcium. Another set of eggs was loaded with Fura-2 and then monitored for 6 h after treatment with T-19, nonimmune IgG, or medium alone. No intracellular calcium oscillations were seen in any of these groups (Fig. 9B). Because the Fura-2 signal fades over time during long-term monitoring, groups of eggs also were treated with the antibody for 6 h and then loaded with Fura-2. These eggs, which were imaged beginning 6 h after the initiation of the antibody treatment, also did not show any intracellular calcium oscillations (Fig. 9C). In all treatment groups, there was a gradual increase in intracellular calcium over the course of the long-term monitoring period; this increase was not different between any of the groups. When eggs were incubated for 6 h in the antibodies before beginning calcium monitoring, the calcium levels during the first 3 h of monitoring were similar to those seen when eggs were monitored from the beginning of antibody treatment (Fig. 9). This observation suggests that the gradual increase in calcium level after 3 h of calcium imaging was a side effect of the monitoring procedure itself, possibly due to accumulated damage from long-term light exposure. The failure to see calcium oscillations was not a technical limitation of the long monitoring time because the eggs displayed calcium oscillations in response to activation with strontium chloride after 6 h of monitoring (Fig. 9B). However, groups of the same T-19-treated eggs that failed to show intracellular calcium increases still displayed the morphological changes characteristic of T-19 treatment.

An alternative explanation for our findings was that calreticulin antibody treatment initiated a transmembrane signal that bypassed calcium oscillations to induce cell cycle resumption. Candidate enzymes that could mediate this process included protein kinase C, phosphatidyl inositol 3-kinase, and MAP kinase. Inhibitors of these three kinases,

Table 3
Summary of actin configuration of eggs treated with anticalreticulin antibodies or nonimmune IgG

Antibody	Concentration (mg/ml)	Actin configuration		Total evaluated	% symmetric
		Symmetric[a] (no actin cap)	Asymmetric[b] (actin cap)		
T-19	5	27	0	27	100
	2	15	0	15	100
	1	2	9	11	18
	0.5	0	22	22	0
C-17	5	15	0	15	100
	2	11	1	12	92
	1	0	9	9	0
	0.5	0	25	25	0
Goat IgG	5	0	18	18	0
	2	0	10	10	0
	1	0	13	13	0
	0.5	0	18	18	0

[a] Symmetric distribution of actin corresponding to that seen in Fig. 10B.
[b] Asymmetric distribution of actin, with an actin "cap" over the metaphase chromatin, similar to image in Fig. 10A.

including BIM (protein kinase C inhibitor), LY 294002 (phosphatidyl inositol 3-kinase inhibitor), and PD 98059 (MAP kinase inhibitor) were tested for their ability to inhibit cell cycle resumption in the treated eggs. Despite incubating the eggs before and during antibody treatment in the presence of concentrations of these inhibitors previously demonstrated to be effective in inhibiting the respective kinase activities in mammalian eggs (Faerge et al., 2001; Shimada and Terada, 2001; Viveiros et al., 2001), no inhibition of cell cycle resumption in response to calreticulin antibody treatment was observed (data not shown).

However, clear alterations in the pattern of microfilament staining were observed when treated eggs were stained with phalloidin to label F-actin (Fig. 10). Metaphase II eggs normally have a distinct actin "cap" overlying the region of the egg cortex containing the metaphase spindle. Calreticulin antibody treatment resulted in a redistribution of the actin such that it was localized evenly throughout the subplasma membrane region, and the actin cap was lost. This response to the calreticulin antibody occurred in a concentration-dependent fashion (Table 3), and was closely correlated with the induction of cell cycle resumption. These results indicate that the signaling pathway induced by the calreticulin antibodies resulted in both cell cycle resumption and reorganization of cytoskeletal structures, without any clear alterations in intracellular calcium.

Discussion

We have demonstrated that the amount of calreticulin at the cell surface and in the perivitelline space increases at fertilization, similar to the pattern seen in the hamster (Munoz-Gotera et al., 2001). Because of the presence of calreticulin in the culture medium after hamster egg activation, it was suggested that calreticulin was found in cortical granules (Munoz-Gotera et al., 2001). However, high-resolution confocal imaging of mouse eggs costained for both cortical granules and calreticulin did not demonstrate colocalization, and in fact, showed localization of calreticulin to the cortical granule-free domain in the region of the metaphase II spindle (Fig. 4). Therefore, the source of the new surface/perivitelline space calreticulin after egg activation is likely to be secretory vesicles present in the cortical region of eggs that are distinct from cortical granules. This conclusion is consistent with previous electron microscopy studies of the mouse oocyte cortical region showing membrane-bound cortical vesicles distinct from cortical granules (Ducibella et al., 1988). Furthermore, these findings are consistent with recent data demonstrating that GM130, a putative Golgi matrix protein, colocalizes with ER vesicle export sites and calreticulin in the mouse egg cortex, but not with cortical granules (Payne and Schatten, 2003).

In response to egg activation, a structure known as the "cortical granule envelope" forms in the perivitelline space and persists throughout preimplantation embryo development (Dandekar and Talbot, 1992). The cortical granule envelope likely is comprised of material secreted from the oocytes during synthesis of the ZP, material secreted from cortical granules during oocyte maturation and at the time of egg activation, and possibly material secreted from ER-associated vesicles, as outline above. Our work (Fig. 3), combined with previous data in the hamster and mouse (Calvert et al., 2003; Munoz-Gotera et al., 2001), shows that calreticulin appears to be one component of this envelope. There also is evidence that several other molecular chaperones that are normally resident in the ER, including HSP90, GRP78, and gp96, are found on the mouse egg surface, and at least one of these, HSP90, increases in amount after egg activation (Calvert et al., 2003). Taken together, these findings suggest that fertilization is associated with exocytosis of material from intracellular vesicles that originate from the ER, in addition to cortical granules, and that this material becomes associated with the cortical granule envelope.

There is little information available regarding the function of the mammalian cortical granule envelope. In the hamster, there is evidence that two cortical granule envelope proteins, known as p62/p56 based on apparent molecular weight, play a role in preimplantation embryo development (Hoodbhoy et al., 2000, 2001). It is possible based on size and immunolocalization that the p62 protein could be calreticulin. In fact, two groups have reported that calreticulin has two forms that resolve on SDS gels at apparent M_r of 62 and 52 kDa (Kwon et al., 2000; Zhu et al., 1997), similar to the proteins identified in the hamster cortical granule envelope. It also has been proposed that the cortical granule envelope functions in the block to polyspermy (Talbot and Dandekar, 2003). If, as our data suggests (Fig. 5), egg surface calreticulin participates in sperm–egg binding, then sperm binding to excess calreticulin in the perivitelline space after fertilization could provide one mechanism to explain this aspect of the polyspermy block.

The amount of calreticulin at the mouse egg surface and in the perivitelline space increases after fertilization, but it clearly is present before fertilization as well. We originally hypothesized that this egg surface calreticulin was involved in egg activation at fertilization. Our results showing that anticalreticulin antibodies partially inhibit sperm–egg plasma membrane binding (Fig. 5) suggest that calreticulin on the unfertilized egg surface may be in proximity to actual sperm binding sites. Alternatively, sperm may bind to calreticulin itself but the antibody may not completely block sperm-binding epitopes. However, given that the inhibition of binding was not robust, and there was no inhibition of sperm–egg fusion, we cannot conclude that calreticulin is directly involved in sperm–egg interactions. One possibility is that a calreticulin-associated protein serves in this capacity.

Although we did not set out to examine the effects of cell surface calreticulin ligation on mouse eggs, it became apparent during our experiments that the eggs were exhibiting morphological changes in response to the presence of the antibody in the culture medium. This finding suggested

that aggregation of the protein was inducing a transmembrane signaling response. Similarly, ligation of calreticulin by anticalreticulin antibodies induces a transmembrane signal to induce calcium fluxes in human neutrophils (Ghiran et al., 2003). Although we used higher concentrations of the primary antibody than were used in the neutrophil experiments, the calreticulin antibody in that case was cross-ligated by a secondary antibody, and hence a much lower concentration of the primary antibody was required. Primary anticalreticulin antibodies, without use of a secondary antibody, also have been shown to induce integrin-dependent platelet aggregation (Elton et al., 2002). In our experiments, at least 0.5 mg/ml antibody was required for a response to be seen, and a complete response was observed with concentrations of 2–5 mg/ml (Table 1). This high antibody concentration was probably necessary because access to the perivitelline space of ZP-intact eggs is limited, unlike the exposed plasma membranes of tissue culture cells.

Our data clearly demonstrates that calreticulin and/or calreticulin-associated proteins present on the egg's extracellular surface before fertilization are capable of transmitting information from the extracellular space to the mouse egg actin cytoskeleton (Table 3; Fig. 10). Similarly, in endothelial cells, engagement of extracellular calreticulin by thrombospondin induces focal adhesion disassembly, a process dependent on the actin-based cytoskeletal network (Goicoechea et al., 2000). On the surface of lung alveolar cells, ligation of calreticulin by the aggregated surfactant proteins found on apoptotic cells leads to phagocytosis of the cellular debris (Gardai et al., 2003), a process requiring signaling to the cytoskeleton. An intriguing possibility is that a similar response mediated by egg surface calreticulin may lead to engulfment of the sperm at fertilization.

In response to ligation of egg surface calreticulin, cell cycle resumption occurred as demonstrated by alterations in DNA configuration and decreases in the activities of cdc2/cyclin B1 and MAP kinase (Figs. 6–8; Table 2). This effect on the cell cycle is similar to that seen in fibroblasts that have a mitogenic response to the binding of the Bβ chain of fibrinogen to calreticulin (Gray et al., 1995). It is unclear what cellular signaling pathway is triggered during the egg cell cycle resumption. The most obvious candidate would be an elevation of intracellular calcium as this cation is responsible for initiating the resumption of meiosis at fertilization, and calcium fluxes are frequently seen in signaling pathways involving calreticulin (Coppolino et al., 1997; Gudz et al., 2002; John et al., 1998; Kwon et al., 2000; Liu et al., 1994). However, continuous observation of these calreticulin antibody-treated eggs did not reveal alterations in intracellular calcium (Fig. 9). Consistent with the lack of evidence for calcium signaling, there was no decrease in the intensity of cortical granule staining in eggs that had undergone cell cycle resumption (Fig. 6). Although unlikely, it is formally possible that there were low amplitude or localized fluxes in intracellular calcium levels that were not detected, and did not cause cortical granule exocytosis. Chemical inhibitors of other signaling effectors through which calreticulin has been shown to operate in other cell types, including protein kinase C, phosphatidyl inositol 3-kinase, and MAP kinase, also did not block calreticulin antibody-induced cell cycle resumption. Because of the effects on the actin cytoskeleton, low molecular weight G proteins of the rho family may be involved downstream in this signaling pathway (Hall, 1998; Kumakiri et al., 2003).

The adaptor molecule(s) that mediates calreticulin's association with the egg surface is unknown. Both transmembrane and GPI-linked proteins serve as adaptor proteins for calreticulin in other cell types. For example, in activated human peripheral T lymphocytes, cell surface calreticulin remains associated with the MHC class I complex (Arosa et al., 1999). CD59, a GPI-linked membrane regulator of complement activity, mediates calreticulin's association with the plasma membrane of circulating neutrophils (Ghiran et al., 2003). Interestingly, treatment of mouse eggs with phosphatidyl inositol-specific phospholipase C, which releases GPI-linked proteins from the cell surface (Low and Finean, 1978), inhibits sperm–egg binding and fusion (Coonrod et al., 1999). Furthermore, mice that cannot express GPI-linked proteins on the egg surface (due to an oocyte-specific conditional knockout of *Pig-a*, a gene essential for GPI anchor biosynthesis) are infertile, and although the eggs are ovulated and appear normal, they cannot be fertilized in vitro (Alfieri et al., 2003). These findings strongly suggest that proper sperm–egg interactions at fertilization depend on the presence of GPI-linked egg surface proteins, and possibly on the presence of other proteins, such as calreticulin, that might be tethered to the plasma membrane via an interaction with GPI-linked proteins.

Specific questions that remain to be answered involve the nature of the adaptor protein(s) that maintains the association of calreticulin with the egg surface, and the signaling pathways activated during calreticulin ligation. In addition, we are investigating the possibility that there is a role for calreticulin in the formation of a tetraspanin web on the egg surface that could be involved in egg activation at fertilization.

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